BIOLOGY LETTERS

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Journal:	*Biology Letters*
Manuscript ID	RSBL-2020-0863.R1
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	de Castro, Erika; University of Cambridge Department of Zoology, Musgrove, Jamie; Smithsonian Tropical Research Institute Bak, Soren; University of Copenhagen Faculty of Science, Department of Plant and Environmental Sciences McMillan, W.; Smithsonian Tropical Research Institute, Jiggins, Chris; University of Cambridge, Department of Zoology
Subject:	Evolution < BIOLOGY, Ecology < BIOLOGY, Biochemistry < BIOLOGY
Categories:	Evolutionary Biology
Keywords:	Heliconius, Passiflora, Cyanogenic glucosides, Coevolution, Lepidoptera, Plant-insect interactions

Author-supplied statements

Relevant information will appear here if provided.

Ethics

Does your article include research that required ethical approval or permits?:
This article does not present research with ethical considerations

Statement (if applicable):
CUST_IF_YES_ETHICS :No data available.

Data

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Statement (if applicable):
The raw data of of this study, including chemical data, is available on Dryad:
de Castro et al. (2020), Phenotypic plasticity in chemical defence allows butterflies to diversify host use strategies, Dryad, Dataset, https://doi.org/10.5061/dryad.gxd2547hh.

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https://datadryad.org/stash/share/311hywgi4WOeJcLuNhR_OcfTDQEeblVFnXZg3EHCyKk.

Conflict of interest

I/We declare we have no competing interests

Statement (if applicable):
CUST_STATE_CONFLICT :No data available.

Authors' contributions

This paper has multiple authors and our individual contributions were as below

Statement (if applicable):
EC - experimental design, data analyses and writing
JM - data collection and writing
SB - data analyses and writing
OM - data collection and writing
CJ - experimental design, data analyses and writing

Phenotypic plasticity in chemical defence of butterflies allows usage of diverse host plants

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Abstract

Hostplant specialization is a major force driving ecological niche partitioning and diversification in insect herbivores. The cyanogenic defences of *Passiflora* plants keep most herbivores at bay, but not the larvae of *Heliconius* butterflies, which can both sequester and biosynthesize cyanogenic compounds. Here, we demonstrate that both *Heliconius cydno chioneus* and *H. melpomene rosina* have remarkable plasticity in their chemical defence. When feeding on *Passiflora* species with cyanogenic compounds that they can readily sequester, both species downregulate the biosynthesis of these compounds. In contrast, when fed on *Passiflora* plants that do not contain cyanogenic glucosides that can be sequestered, both species increase biosynthesis. This biochemical plasticity comes at a fitness cost for the more specialist *H. m. rosina*, as adult size and weight for this species negatively correlate with biosynthesis levels, but not for the more generalist *H. c. chioneus*. In contrast, *H. m rosina* has increased performance when sequestration is possible on its specialised hostplant. In summary, phenotypic plasticity in biochemical responses to different host plants offers these butterflies the ability to widen their range of potential hosts within the *Passiflora* genus, while maintaining their chemical defences.

INTRODUCTION

Hostplant specialization is undoubtedly one of the most important forces driving diversification and shaping niche dimension for phytophagous insects [5][6][7]. Most specialized insects have not only evolved the ability to handle the chemical defences of their favourite hosts and grow despite them, but have often become dependent on these compounds [8].Hence, the majority of toxic insects rely on plant compounds to protect them against predators and pathogens[14]. Sequestration of plant toxins is an adaptation that arose in several insect orders, most notably Coleoptera and Lepidoptera, playing an important role in the antagonistic coevolution with their hosts[15][16]. However, whereas inducible defences of plants by herbivory have been well studied [9][10][11][12], there has been relatively limited exploration of the mechanisms of biochemical plasticity in insects that could allow them to exploit diverse hosts [13]

Although sequestration considerably increases fitness of specialized insect herbivores on their preferred diet, it has subordinated their toxicity and niche breadth to specific plant taxa. Arguably, the escalation of diet specialization could lead to an evolutionary and ecological "dead end" [17][18]. Phenotypic plasticity is widely recognised as an adaptation that allows organisms to survive in a variable environment [1]. Furthermore, plasticity in the origin of chemical defences might permit populations to colonize otherwise inaccessible niches or habitats, providing new targets for evolutionary process [2][3][4]. In contrast to most aposematic insects, *Heliconius* butterflies have both diet-acquired (sequestered) and autogenous (biosynthesized) chemical defences, which makes them a suitable system to explore the correlation between biochemical plasticity and diet specialization.

Heliconius biosynthesize aliphatic cyanogenic glucosides (CNglcs) from the amino acids valine and isoleucine [19]. Their obligatory *Passiflora* hosts are also chemically defended by a broad range of CNglcs [20], several of which are sequestered by *Heliconius* during larval feeding [21][22][23] (Table S1). It has been suggested that *Heliconius* species specialized for sequestration show reduced biosynthesis [23][24]. However, it remains unknown whether there is within-species plasticity in the use of sequestered versus autogenous toxicity, as this is a poorly understood phenomenon in aposematic insects. Switching between biosynthesis and sequestration of toxins could allow insects to colonise a wider array of potential host plants independently of sequestration, while also maintaining their chemical defences.

Here, we explore the trade-off between biosynthesis and sequestration of toxins within two *Heliconius* species with different host-use strategies to answer the following questions: 1) Is there plasticity in the adoption of biosynthesis and sequestration on different host plants? 2) Does biochemical plasticity

have a fitness cost? 3) Is this cost similar for insects with generalist and specialist hostplant preferences? To answer these questions, we raised the sympatric butterflies *Heliconius melpomene rosina* and *Heliconius cydno chioneus* on four *Passiflora* species with varied CNglc profiles (Table S1). It has been reported that although their larvae perform well on several hosts, *H. m. rosina* has strong oviposition preferences for *P. menispermifolia*, whereas *H. c. chioneus* oviposits on many *Passiflora* plants [25]. Here, we measured size, weight and CNglc content of adults raised on different larval diets to investigate whether there were possible fitness trade-offs when feeding on different plants or adopting different chemical defence strategies.

METHODS

Butterfly rearing

Butterflies were reared at the Smithsonian Tropical Research Institute, Panama. Stocks of *H. cydno chioneus* and *H. melpomene rosina* were maintained in cages and fed *ad libidum* with flowers (*Psiguria triphylla*, *Gurania eriantha*, *Psychotria poeppigiana*, *Lantana sp.*) and artificial nectar (10% sugar solution). Plants of one of the four species used in the experiment - *P. biflora, P. menispermifolia*, *P. platyloba*, and *P. vitifolia* - were always kept in cages for oviposition. Eggs were collected daily and kept in closed tubs until hatching. On the morning of hatching, larvae were transferred to treatment-specific cages onto individual shoots. Cages were checked daily and fresh sterilized shoots provided regularly. Pupae were immediately removed, weighed the day after pupation and taped inside individual 350 ml tubes. Butterfly measurements were acquired few hours after eclosion. Body length was measured from the end of the head to the end of the abdomen and forewing length was measured from the central base to the most distal point. Butterflies were added into tubes containing 1.5 mL methanol 80% (v/v) and stored at 4 °C.

Chemical Analyses

Samples were homogenized in 1.5 mL methanol 80% (v/v) where they were soaked and centrifuged at 10,000 x g for 5 min. Supernatants were collected and kept in HPLC vials at -20 °C. Sample aliquots were filtered (Anapore 0.45 µm, Whatman), diluted 50X times (v/v) and injected into an Agilent 1100 Series LC (Agilent Technologies, Germany) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics). Chromatographic separation was carried out using a Zorbax SB-C18 column (Agilent; 1.8µM, 2.1x50mm). MS and LC conditions are described in [23]. Sodium adducts of CNglcs detected in the butterflies were identified by comparing their m/z fragmentation patterns and RTs to authentic standards [20] and quantified as described in [23].

Statistical Analyses

Statistical analyses were performed using R version 3.5.1 (R Core Team, 2017). ANOVA followed by Tukey HSD was used to analyse the effects of each diet on the measured traits within species. ANCOVA and linear regressions were used to verify if biosynthesis have similar fitness cots for butterflies with generalist and specialist hostplant preferences (See details in Supplementary Material).

RESULTS

Larval diet affected the CNglc profile of both *H. melpomene* and *H. cydno* butterflies (Figure 1). Both species sequestered deidaclin when fed on *P. menispermifolia*, although *H. melpomene* sequestered significantly more deidaclin than *H. cydno* (ANOVA, $F_{1,22}$= 8.851; p= 0.00699). In both species, Deidaclin sequestration from *P. menispermifolia* was associated with a reduction of biosynthesis in comparison with other diets. The modified CNglc passibiflorin from *P. biflora* and tetraphyllin B-sulphate from *P. vitifolia* were not found in either butterfly species raised on these diets, suggesting that they cannot sequester these compounds. Surprisingly, traces of prunasin recently found in the haemolymph of larvae raised on *P. platyloba* [22] were not present in adults of either butterfly species.

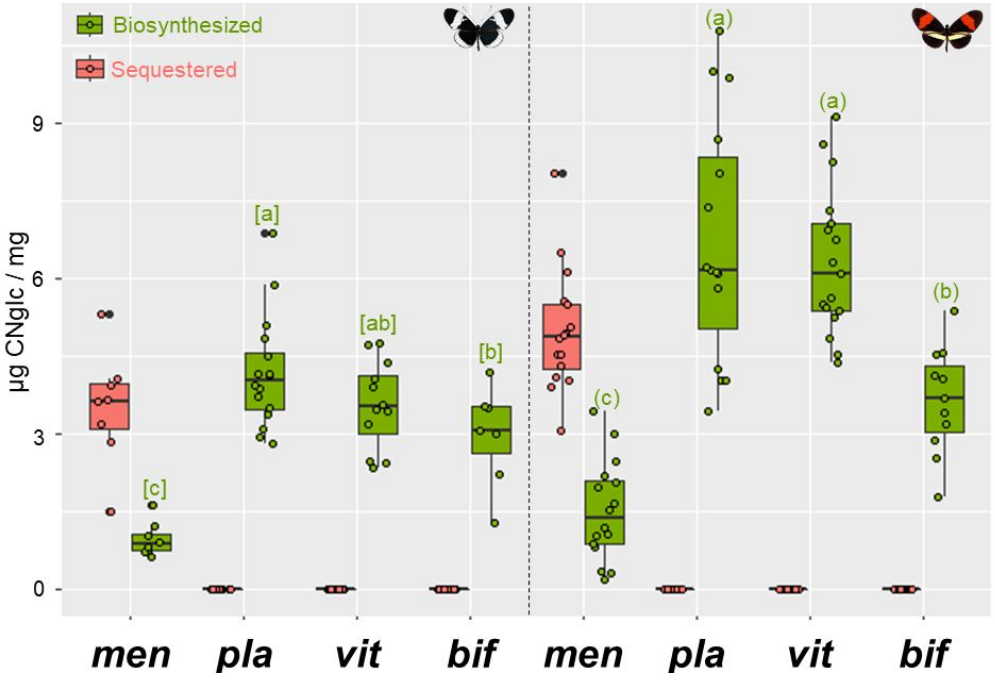
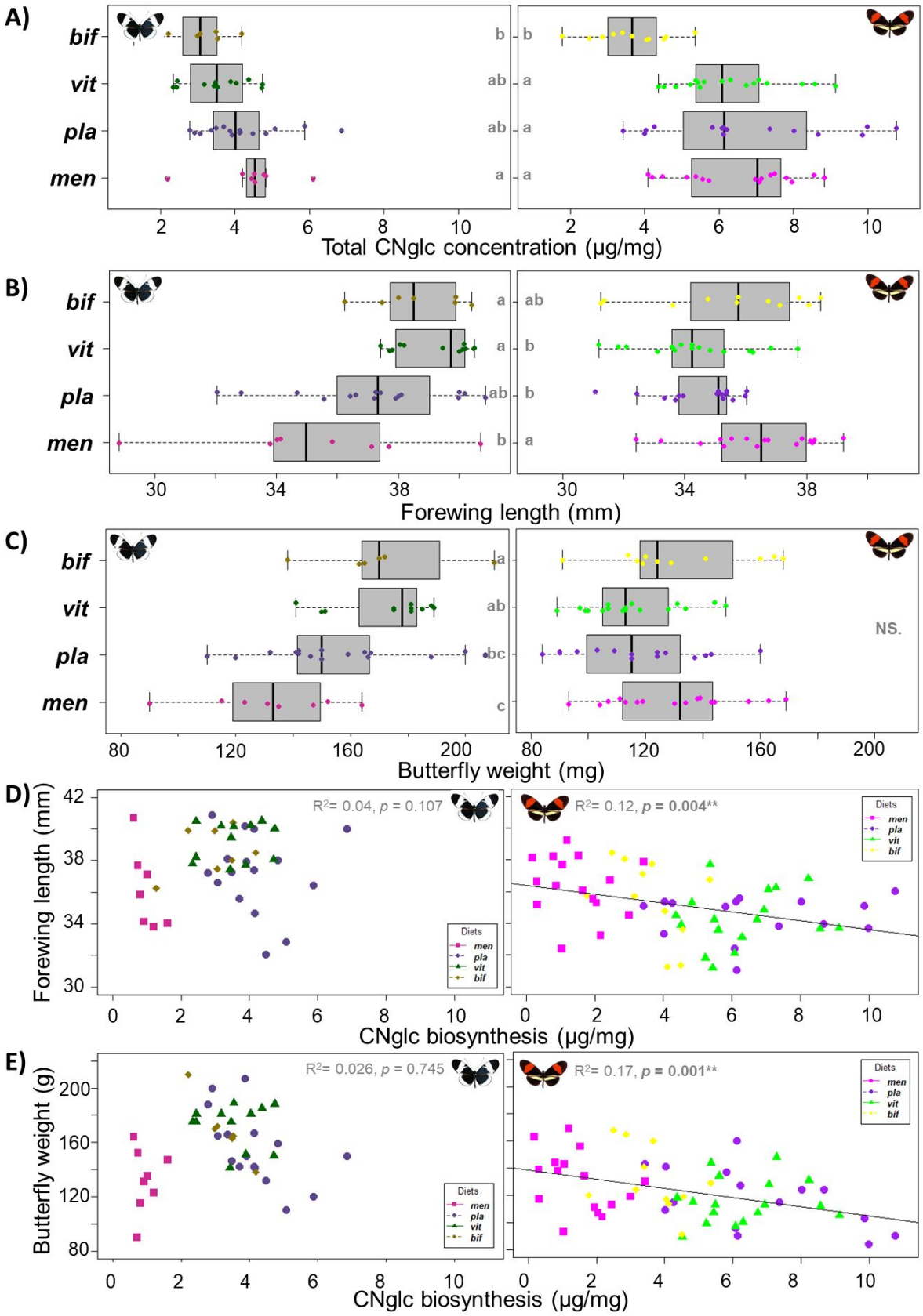


Figure 1. CNglc composition of *H. cydno* (left, N= 39) and *H. melpomene* (right, N= 55) raised on different *Passiflora* diet. men= *P. menispermifolia*; pla= *P. platyloba*; vit= *P. vitifolia*; bif= *P. biflora* (non-host). Green boxplots correspond to the biosynthesized CNglcs linamarin and lotaustralin found in all butterflies. Letters over boxplots correspond to post-hoc comparisons within butterfly species, where different letters indicate statistically significant concentration of biosynthesized CNglcs. Salmon boxplots to the sequestered CNglc deidaclin only detected in butterflies raised on *P. menispermifolia*. Tetraphyllin B-sulphate, passibiflorinand prunasin were not detected in butterflies, even though they were present in the food plants *P. vitifolia*, *P. biflora* and *P. platyloba*, respectively (Table S1).

Larval diet not only influenced the composition, but also the total CNglc concentration in both species (ANOVA, *H. cydno:* $F_{3,39}$ = 3.653, p= 0.0205; *H. melpomene:* $F_{3,55}$= 8.776, p= 0.00007) (Figure 2A). Both had less CNglcs when reared on *P. biflora,* which they normally do not use as a host. On average, butterflies also had a higher CNglcs content when reared on *P. menispermifolia* than on *P. platyloba* and *P. vitifolia,* though these differences were not statistically significant. CNglc concentrations in *H. cydno* (3.85 ± 1.08) were on average lower than *H. melpomene* (5.96 ± 1.97).

Larval diet also affected size and weight of both species. Forewing size of *H. cydno* (ANOVA, $F_{3,39}$= 5.14; p= 0.004) was larger and more strongly influenced by larval diet than *H. melpomene* ($F_{3,57}$= 4.0; p= 0.012) (Figure 2B). *H. cydno* had larger forewings when fed on *P. vitifolia* and *P. biflora*, and smaller on *P. menispermifolia* and *P. platyloba.* In contrast, adults of *H. melpomene* had larger forewings when reared on *P. menispermifolia* and *P. biflora*, and smaller on *P. vitifolia* and *P. platyloba*. Broadly similar effects of diet were seen for butterfly weight (Figure 2C), although this was not significant for *H. melpomene*. These trends were also similar in other size and weight measurements (Figure S1). Sex differences in forewing size, butterfly weight and total CNglcs concentration were not observed in either species (Table S3).

In order to verify whether biosynthesis versus sequestration plasticity has fitness costs for both species, we performed an ANCOVA analysing the effect of biosynthesized CNglcs and diet on the fitness proxies, size and weight. In the generalist *H. cydno*, even though larval diet strongly affects forewing size ($F_{3,35}$= 3.7514 p= 0.0195) and butterfly weight ($F_{3,35}$ = 16.222 p= 0.000001), this effect is not correlated with whether they sequester or biosynthesize CNglcs (forewing size: $F_{1,35}$=3.1465 p= 0.0848; butterfly weight: $F_{1,35}$= 0.044 p = 0.8351) (Figure 2D and 2E). Thus, although larval diet has a profound effect on *H. cydno* fitness, this is not caused by the CNglc composition of the plants but by their other nutritional properties. Whilst, in the ecological specialist *H. melpomene,* there is a negative effect of CNglc biosynthesis on forewing size ($F_{1,51}$= 9.1370, p= 0.0039)(Figure 2D) and butterfly weight ($F_{1,51}$= 11.8676, p= 0.0011)(Figure 2E), and the effect of diet is not significant in this correlation (forewing size: $F_{3,51}$= 1.1321, p= 0.3449; butterfly weight: $F_{3,51}$= 0.5701, p= 0.6372). This suggests that despite their successful performance on many *Passiflora* diets, CNglc biosynthesis has a fitness cost for *H. melpomene rosina*, which mostly lay eggs on *P. menispermifolia* from which they can sequester CNglcs.



120

121 **Figure 2.** Effect of larval diet on **A)** total CNglc concentration; **B)** forewing length and **C)** butterfly
122 weight of *H. cydno* (left, N= 39) and *H. melpomene* (right, N= 55). Letters over boxplots correspond to
123 post-hoc comparisons within butterfly species, where different letters indicate statistically significant
124 treatments. Correlation between concentration of biosynthesized CNglcs (accounting for diet) **D)** and

125 forewing length; **E)** and butterfly weight. pla= men= *P. menispermifolia*; *P. platyloba;* vit= *P. vitifolia;*
126 bif= *P. biflora* (non-host).
127

128 **DISCUSSION**

129 We documented, for the first time, intra-specific plasticity in the CNglc profile of both *H. melpomene*
130 *rosina* and *H. cydno chioneus* in response to larval diet (Figure 1). When reared on a plant with
131 cyclopentenyl CNglcs that can be sequestered, both species invest less in biosynthesis of aliphatic
132 CNglcs, a trade-off that has previously been observed between different species[26][23]. This
133 plasticity should enable *Heliconius* to exploit different *Passiflora* hosts – independently of plant CNglc
134 composition – as they can maintain their defences through biosynthesis when sequestration is not
135 possible. Interestingly, many *Passiflora* species seem to have modified their CNglcs to prevent
136 sequestration by heliconiines [23]. Here, we show that the two modified CNglcs passibiflorin and
137 tetraphyllin-B sulphate were not sequestered by either *Heliconius* species, suggesting *an evolutionary*
138 *arms-race between the plants and their herbivores.* For both *Heliconius* species, individuals raised on
139 their natural host range reached a similar total concentration of CNglcs regardless of how they
140 acquired their cyanogenic defencesA similar pattern has been observed in the moth *Zygaena*
141 *filipendulae*, another rare example of lepidopteran that can both *de novo* biosynthesize and sequester
142 the same defence metabolites [27]. *Z. filipendulae* balance their cyanogenic content with biosynthesis
143 when sequestration is not possible, however at the detriment of growth [28][29]. It is likely that, as in
144 *Zygaena* moths, *Heliconius* have adaptations to optimize the energetic cost of their toxicity:
145 decreasing biosynthesis of CNglcs when these compounds are available for sequestration and
146 increasing it when they are not.

147 Balancing biosynthesis and sequestration in response to diet is not exclusive to Lepidoptera. For
148 example: *Chrysomela lapponica* larvae (Coleoptera) increase 40 fold synthesis of defensive esters
149 when effective sequestration of salicylic glycoside is not possible [30]. When raised on milkweed,
150 *Lygaeus equestris* (Heteroptera) sequester cardenolides and reduce biosynthesis of volatile defences
151 in their scent-gland in comparison to bugs fed sunflower seeds (no cardenolides)[31]. Even though in
152 these examples autogenous and sequestered defence compounds belong to completely divergent
153 chemical classes and are likely under different selection forces, there is still a trade-off between
154 biosynthesis and sequestration. This emphasizes the complexity of biochemical plasticity in insects in
155 response to diet and suggest that this process may be of greater importance than currently realized.

156 Biochemical plasticity could be advantageous if, for example, hostplants are very heterogenous in
157 chemical content or of it enables insects to use a broader range of hostplants. Avoidance of
158 interspecific competition is possibly the major force shaping the evolution of hostplant range for

Heliconius in Panama, where coexisting species rarely share oviposition preference for the same *Passiflora*[34][35]. Biochemical plasticity could therefore be associated with a wide range among of *Passiflor*a hosts, allowing the coexistence of multiple *Heliconius* species and enable them to further diversify and/or switch their use of *Passiflor*a species while maintaining their chemical defences. Nevertheless, the cost of biosynthesis versus sequestration and diet plasticity seems to vary between *Heliconius* species

In *Heliconius*, recent studies have also shown that some monophagous species have become more efficient in sequestration and might have lost their biosynthetic ability [22][37]. Here, we show that although the ability to shift between chemical strategy is present in two closely related species, the cost of doing so differs. Although larval diet has a stronger effect on the performance of the more generalist *H. cydno*, fitness costs of biosynthesis per se was only observed for the more specialist *H. melpomene* (Figure 2D and 2E). Hence, although the phenotypic expression is plastic and varies with hostplant diet, it does so within a constrained range that is likely genetically defined. A new study has demonstrated that there is substantial intraspecific variation in the ability of these butterflies to biosynthesize CNglcs and suggested a genetic component to this variation [37]. Together with our results, this suggest that genetics and phenotypic plasticity play an important role in how aposematic herbivores balance autogenous versus acquired defences, the evolution of diet breadth, and in the coevolution with their hosts plants.

It has been suggested that plasticity might facilitate the invasion of new habitats and therefore evolutionary innovation [4][36] . It seems likely that biochemical plasticity originally evolved in species such as *H. cydno* as an adaptation to facilitate a wide host plant range, but might also enable *Heliconius* to further diversify and/or switch their use of *Passiflor*a species while maintaining their chemical defences. Plasticity can therefore be seen as both a potential cause and a consequence of hostplant use diversification, but it is difficult to tease apart these two factors in this particular case.

For many decades, specialized insects were thought to have a simple biochemical machinery, sequestering from plants and becoming subordinated to them. This has contributed to the hypothesis that diet specialization would often led to an evolutionary and ecological “dead end”. With the advances of analytical chemistry and metabolomic approaches, we are now seeing that many insects can biosynthesize specialized metabolites [30][31], modify plant-acquired compounds[39] and even recycle them[29]. Our findings highlight that biochemical plasticity is not only possible, it may be more prevalent than currently assumed, and it may have far reaching consequences for diet breadth, ecological niche partitioning and speciation.

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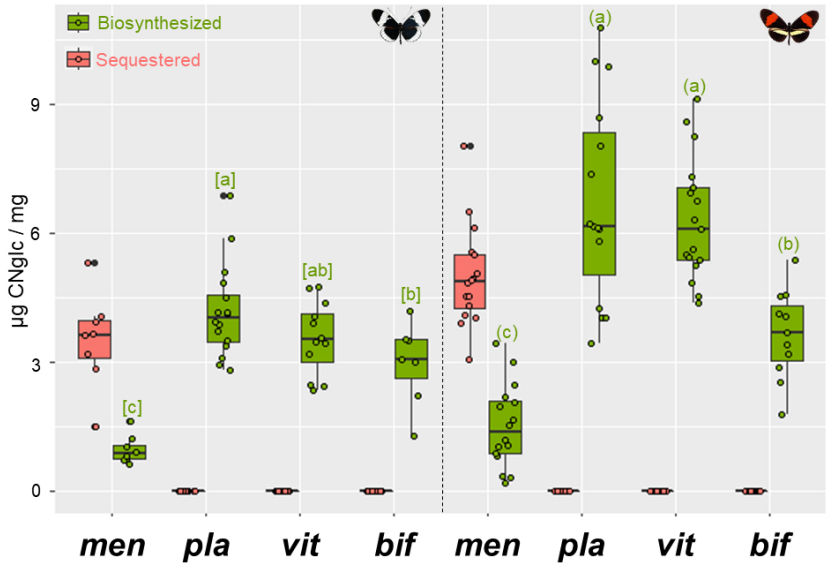

Figure 1. CNglc composition of H. cydno (left, N= 39) and H. melpomene (right, N= 55) raised on different Passiflora diet. men= P. menispermifolia; pla= P. platyloba; vit= P. vitifolia; bif= P. biflora (non-host). Green boxplots correspond to the biosynthesized CNglcs linamarin and lotaustralin found in all butterflies. Letters over boxplots correspond to post-hoc comparisons within butterfly species, where different letters indicate statistically significant concentration of biosynthesized CNglcs. Salmon boxplots to the sequestered CNglc deidaclin only detected in butterflies raised on P. menispermifolia. Tetraphyllin B-sulphate, passibiflorinand prunasin were not detected in butterflies, even though they were present in the food plants P. vitifolia, P. biflora and P. platyloba, respectively (Table S1).

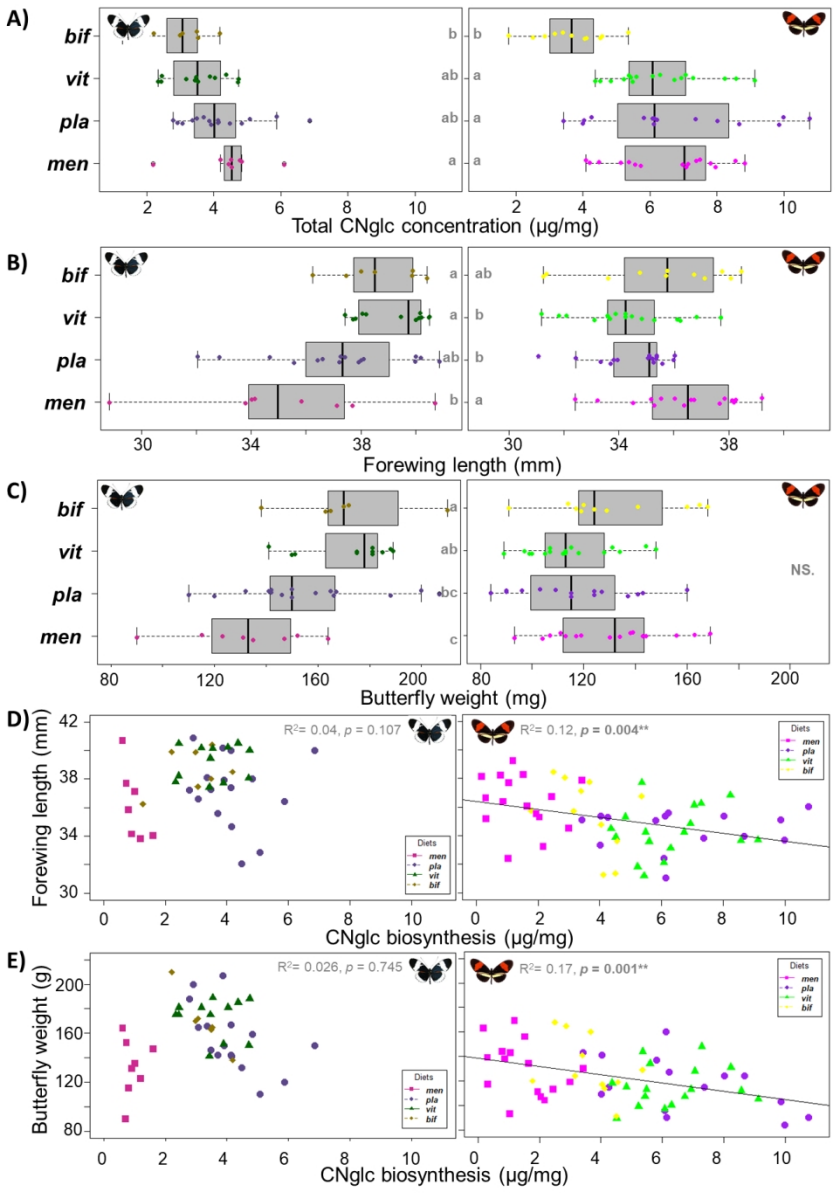

Figure 2. Effect of larval diet on A) total CNglc concentration; B) forewing length and C) butterfly weight of H. cydno (left, N= 39) and H. melpomene (right, N= 55). Letters over boxplots correspond to post-hoc comparisons within butterfly species, where different letters indicate statistically significant treatments. Correlation between concentration of biosynthesized CNglcs (accounting for diet) D) and forewing length; E) and butterfly weight. pla= men= P. menispermifolia; P. platyloba; vit= P. vitifolia; bif= P. biflora (non-host).